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1 # Effect of β-adrenergic receptor agents on cardiac structure and function
2 # and whole-body gene expression in *Daphnia magna*

3

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19 **Abstract**

20

21 Propranolol (PRO), a human β-AR (β-adrenergic receptor) antagonist, is considered to result
22 in specific effects in a non-target species, *D. magna,* based on our previous studies. The
23 present study investigated the effects of β-AR agents, including an antagonist and agonist
24 using pharmacologically relevant endpoints as well as a more holistic gene expression
25 approach to reveal the impacts and potential mode of actions (MOAs) in the model non-target
26 species. Results show that the responses in cardiac endpoints and gene expression in *D.*
27 *magna* are partially similar but distinguishable from the observations in different organisms.
28 No effect was observed on heart size growth in PRO and isoprenaline (ISO) exposure. The
29 contraction capacity of the heart was decreased in ISO exposure, and the heart rate was
30 decreased in PRO exposure. Time-series exposures showed different magnitudes of effect on
31 heart rate and gene expression dependent on the type of chemical exposure. Significant
32 enrichment of gene families involved in protein metabolism and biotransformation was
33 observed within the differentially expressed genes, and we also observed differential
34 expression in juvenile hormone-inducible proteins in ISO and PRO exposure, which is
35 suspected of having endocrine disruption potential. Taken together, deviation between the
36 effects of PRO and ISO in *D. magna* and other organisms suggests dissimilarity in MOAs or
37 attributes of target bio-molecules between species. Additionally, PRO and ISO may act as
38 endocrine disruptors based on the gene expression observation. Results in the present study
39 confirm that it is challenging to predict ecological impact of active pharmaceutical
40 ingredients (APIs) based on the available data acquired through human-focused studies.
41 Furthermore, the present study provided unique data and a case study on the impact of APIs
42 in a non-target organism.

43

44 *Keywords*

45 Propranolol, cardiac endpoints, gene expression, endocrine disruption, *Daphnia magna*

46

47 Main Finding

48 • Present study reveals that effects of β-adrenergic agents on heart function and gene
49 expression of *Daphnia magna* are distinguishable from effects on other species despite partial
50 similarity.

51

52

53 **1. Introduction**

54

55 APIs that are introduced into the aquatic environment can affect non-target organisms and,
56 consequently, result in unintended harmful effects on ecosystems. Unexpected detrimental
57 effects of APIs in natural ecosystems have been reported and are a pressing concern in
58 environmental science (Brodin et al., 2013; Cuthbert et al., 2006; Pérez et al., 2012). Studies
59 have proven that vertebrate biochemical messengers react with receptors in wildlife, which
60 are potential targets of several APIs and responsible for crucial physiological functions in
61 non-target organisms (Buonomo et al., 1984; Kashian and Dodson, 2004). Although the
62 homology of pharmaceutical target receptors is highly species-dependent, studies have shown
63 therapeutic actions of APIs in non-targeted organisms (Brooks and Huggett, 2012; Campos et
64 al., 2012; Gunnarsson et al., 2008). Considering persistent concentrations of APIs up to
65 several µg/L in the aquatic environment (Tijani et al., 2016), deeper understanding of the
66 ecological risk of APIs is necessary to investigate how the effects of APIs occur and whether
67 those are pharmacologically relevant.

68 PRO, a cardiovascular drug, is one of the potentially harmful APIs to aquatic organisms.
69 Results of our previous study on *D. magna* confirmed a heart-specific action of PRO in a
70 non-target organism (Jeong et al., 2018), but details on the cardiac effects were limited due to
71 the lack of observation on additional pharmacologically relevant endpoints. Furthermore, the
72 complexity of the proposed MOAs of PRO in non-targeted organisms (Huggett et al., 2002;
73 Massarsky et al., 2011) requires a study employing multiple biomarkers to further understand
74 the effects and potential MOAs of the β-AR agent. Therefore, the current study focused on
75 multiple pharmacologically relevant endpoints to investigate how β-AR binding agents affect
76 *D. magna*. In addition, whole-body gene expression profiling in a time-course experiment
77 was performed to provide deeper insight into the effects and potential MOAs. β-AR
78 antagonist and agonist, PRO and ISO (Day and Roach, 1974), were used to activate and

79 deactivate the target receptor. A mixture of the agents was also utilized to confirm a mixture
80 effect as the effect of ISO is abolished by PRO (Hainsworth et al., 1973).
81
82 **2. Materials and Methods**
83
84 *2.1. Preparation of chemical solutions and model organisms*
85
86 (±)-Propranolol hydrochloride and isoprenaline hydrochloride were purchased from Sigma-
87 Aldrich. Reagents were handled as recommended by the manufacturer. Chemical solutions
88 for each exposure test were generated using media identical to the culture media. For the
89 investigation of cardiac functional and structural change, an in-house *D. magna* culture was
90 used. Culturing methods and media composition were in compliance with the US
91 Environmental Protection Agency (EPA) guideline (Weber, 1991). For the gene expression
92 profiling, the Xinb3 genotype of *D. magna* was used, which had been raised in the laboratory
93 of K. D. Schamphelaere (Asselman et al., 2016). COMBO media and Organization for
94 Economic Co-operation and Development (OECD) guidelines were used for the test
95 organism culture (Kilham et al., 1998; OECD, 2012). The Xinb3 isolate was specifically
96 selected for the gene expression profiling because it has been used to develop the recently
97 published transcriptome of *D. magna* and allowed us to easily identify potential targets using
98 the recently published corresponding gene set of *D. magna* (Orsini et al., 2016).
99
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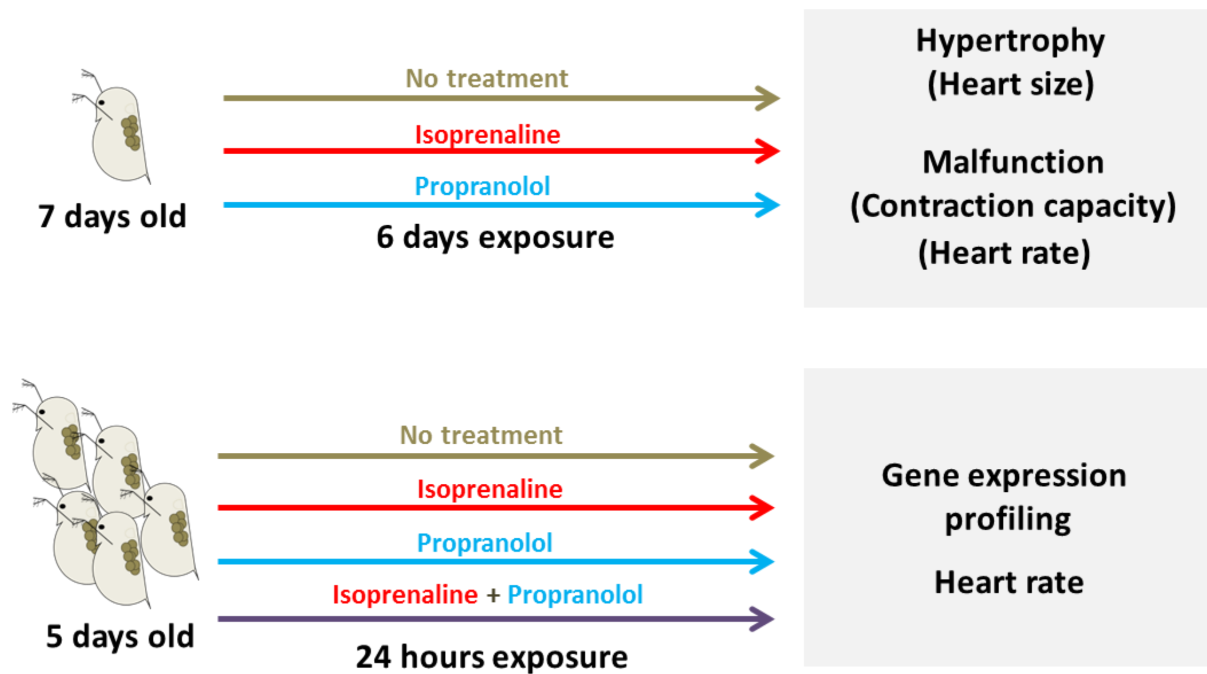

101
102 **Figure 1.** Overall experiment design.
103
104
105 *2.2. Chemical exposure experiments and body sampling*
106
107 The overall design of the exposure experiments is described in Figure 1. The first and
108 second experiments investigated the heart-related endpoints monitoring and whole body gene
109 expression profiling, respectively. In the first experiment, animals were exposed to solutions
110 of PRO and ISO during 6 days to study chronic effects in heart size, contraction capacity, and
111 heart rate. The mixture of PRO and ISO was additionally used to investigate mixture effect of
112 the target compounds on heart rate. The concentrations of solutions were 96 µg/L and 1,755
113 µg/L for PRO and ISO solution, which are sub-lethal concentrations (Dzialowski et al.,
114 2006). Seven-day-old animals were individually exposed to each chemical solution in 30-mL
115 plastic beakers. After 6 days of exposure, cardiac size, body size, and heart volumes in
116 relaxation and contraction states were measured under the microscope. Every exposure was
117 replicated 6 times.
118 In the second experiment, animals were exposed for 24 h to 2 different concentrations of
119 PRO, a single concentration of ISO, and a mixture of PRO and ISO to study changes in gene
120 expression and heart rate. Five-day-old daphnids, which have no egg on their clutches, were
121 used to avoid detection of gene expression in eggs. The solution concentrations were 0.9

mg/L and 3.6 mg/L for PRO solutions, 84.4 mg/L for an ISO solution, and 0.9 mg/L of PRO and 84.4 mg/L of ISO for a mixture. The concentrations in the exposure were chosen to be sufficiently high to observe clear time-series changes of heart rate and corresponding gene expression regulation within 24 h, which is the period of initial response of *D. magna* to the exposed chemicals. Twenty individuals were exposed to different chemical solutions separately in a volume of 35 ml and were harvested at 1, 3, 6, and 24 h of exposure. When the whole body sample was gathered, heart rates were recorded from separate exposure sets, an individual *D. magna* in 20 mL chemical solutions, under a microscope. The body sampling was triplicated, and heart rate measurement was replicated 6 times. Exposure conditions, including room temperature and food concentration, were the same across all exposures and identical to the culturing conditions. Solutions in all exposures were daily generated and renewed daily to prevent degradation of the chemicals.

2.3. Confirmation of chemical concentrations of solutions

Chemical concentrations in the exposure solutions were quantified separately using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in triplicate, and the averages of the estimated concentrations were used in this manuscript. The studies of Jeong et al. (Jeong et al., 2016) and Gu et al. (Gu et al., 2008) were used as references for analysis method development, and metoprolol was used as an internal standard compound. In brief, quantification was performed using a Waters Quattro micro high-performance LC-MS/MS system. Chromatographic separations were performed on an ACQUITY UPLC BEH C18 column (2.1 × 150 mm, 3.5 μM, water). The mobile phases were Milli-Q water (0.1% formic acid) and acetonitrile (0.1% formic acid). The column temperature was 40°C. The mass condition was as follows: ESI positive ion mode; source temperature of 150°C; desolvation temperature of 350°C; desolvation gas flow of 500 L/h; nitrogen gas for desolvation, and argon gas for collision. The instrument was operated in multiple reaction monitoring mode and ion masses of 260>183, 208>166, and 268>74 were used for PRO, ISO, and metoprolol, respectively.

2.4. Heart rate, heart size, and body size measurement from the 6-day exposure set

154 Animals of similar body size were chosen to minimize biological variation. After 6 days of
155 exposure, individuals were placed under a microscope (CKX41SF, Olympus) equipped with
156 a digital camera, and heart rate, heart size, and body size were measured. The heart rate for
157 30 sec was counted for 30s in slow-motion mode using a GOM player (Gretech Corporation),
158 and relative heart rate to control was calculated for comparison between different exposures.
159 The heart size and body size were captured and estimated using ImageJ software, and raw
160 units (pixel) were not transformed to actual-size units (Schindelin et al., 2015). Heart size and
161 body size were measured at the start and at the end of the exposure to calculate the relative
162 change. To compare heart sizes between individuals, the measured heart size was normalized
163 to the measured body size.
164 The contraction capacity was defined as the area deduction between the heart sizes in
165 relaxation and contraction states, and ImageJ was used for the measurements and calculations
166 of the heart area. The details of the contraction capacity calculation are listed in Figure S1.
167
168 *2.5. Gene expression profiling and heart rate measurement from the 24-h exposure set*
169
170 During the 24-h exposure period, the animals were harvested after 1, 3, 6, and 24 h of
171 exposure, at which heart rate was also simultaneously recorded. The measurement method of
172 heart rate was the same as that in the 6-day exposure experiment. Body samples of *D. magna*
173 frozen using liquid nitrogen were stored in 1.5 ml microtube at -80°C until RNA extraction.
174 Total RNA was extracted from the body sample using the RNeasy kit and Qiashredder
175 (Qiagen, Venlo, The Netherlands) following the manufacturer's protocol.
176 Concentration and quality of the extracted total RNA was measured using the Quant-it
177 RiboGreen RNA assay (Life Technologies, Grand Island, NY, US) and using an RNA 6000
178 Pico Chip Kit on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). An
179 Illumina mRNA sequencing library was made from 500 ng of total RNA using the Truseq
180 stranded messenger RNA Library Prep Kit (Illumina, San Diego, CA, US). Libraries were
181 quantified by quantitative polymerase chain reaction, according to Illumina's protocol
182 Sequencing Library qPCR Quantification protocol guide. A DNA 1000 chip (Agilent
183 Technologies, Santa Clara, CA, US) was used to control the library's size distribution and
184 quality. In total, 21 RNA-sequence (RNA-seq) libraries were equimolarly pooled and
185 sequenced on an Illumina NextSeq 500 high throughput run, generating 1 × 75 base pair

reads. All sequencing data was deposited in GEO and is available under accession number GSE104487.

2.6. Data analysis

For the data from cardiac endpoints monitoring, an analysis of variance (ANOVA) test was performed with a *post hoc* Tukey's test to compare the significant changes between exposure sets and control using SPSS 18.0.0 software. For the RNA-seq data, quality of the raw reads was assessed using FastQC (Babraham Institute, Cambridge, UK, version 0.11.5). Potential adapter contamination in the raw reads was removed using Trim Galore (Babraham Institute, Cambridge, UK, version 0.3.2.). Additionally, reads were dynamically trimmed to the longest stretch of bases to obtain at least 99.9% base-call accuracy. Reads were aligned to the Xinb3 transcriptome (Orsini et al., 2016) using Bowtie2 (version 2.1.0) (Langmead and Salzberg, 2012). Aligned reads were processed with HTseq to count the number of reads per gene (Anders et al., 2015). These counts files were then statistically analyzed in R and Bioconductor (Gentleman et al., 2004; Ihaka and Gentleman, 1996) for differential gene expression. Trimmed means of M-values were applied for normalization after data filtration. Quasi-likelihood dispersion was estimated (Lun et al., 2016). Gene expression at different time points and chemical exposures were compared with controls to identify significant differences using factorial designs to determine the effects of exposure time, PRO, ISO, and any potential interactions, e.g., interactions between PRO and time. This was done by fitting a quasi-likelihood negative binomial generalized log-linear model to the data and conducting gene-wise statistical tests for each statistical contrast or coefficient of the log-linear model, which includes both main effects and interaction effects (Lun et al., 2016). The Benjamini-Hochberg method was applied to adjust p values (Benjamini and Hochberg, 1995). Genes with a significant p-value (<0.05) and a positive log2 fold change were identified as significantly upregulated, genes with a significant p-value and a negative log2 fold change significantly downregulated. No additional cut-off value was used. Fisher's exact test was performed to identify enrichment or overrepresentation of gene families and pathways within the differentially expressed gene set (Asselman et al., 2012).

3. Results

219 *3.1. Effect on heart rate, size, and contraction capacity after 6-day exposure*

220

221 The effects of PRO and ISO on cardiac structure and function were determined by
222 measuring heart size, heart contraction capacity, and heart rate (Figure 2). Heart size did not
223 significantly change after exposure to PRO and ISO (Figure 2a). However, heart contraction
224 capacity decreased significantly in ISO exposure (Figure 2b). The heart rate measurement
225 results are shown in Figure 2c. Heart rate was significantly reduced after 6 days of PRO
226 exposure, whereas it was not affected by ISO. Interestingly, when ISO was mixed with PRO,
227 the heart rate was significantly higher than when *D. magna* was exposed to PRO alone. The
228 lowered heart rate in the mixture was still significantly decreased from that of the control.

229

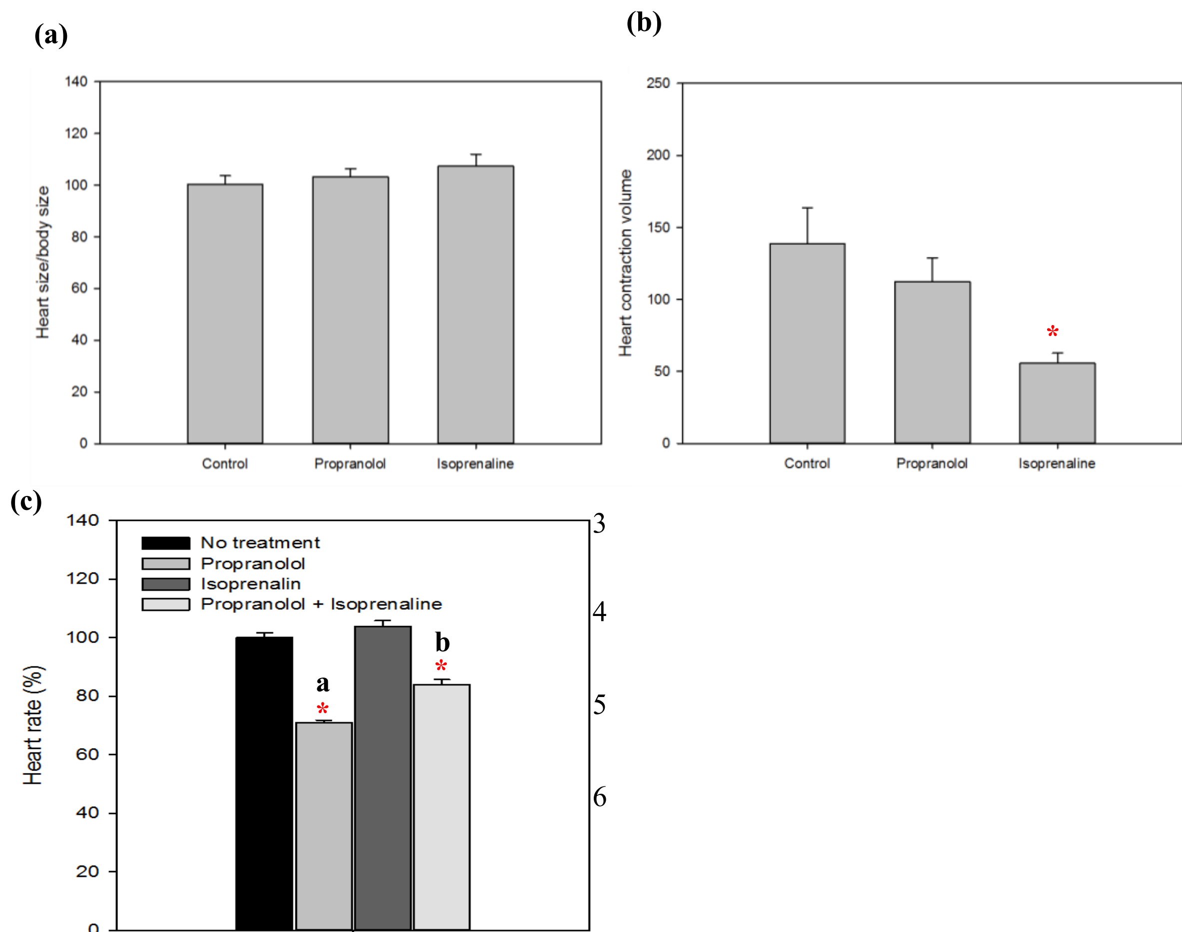

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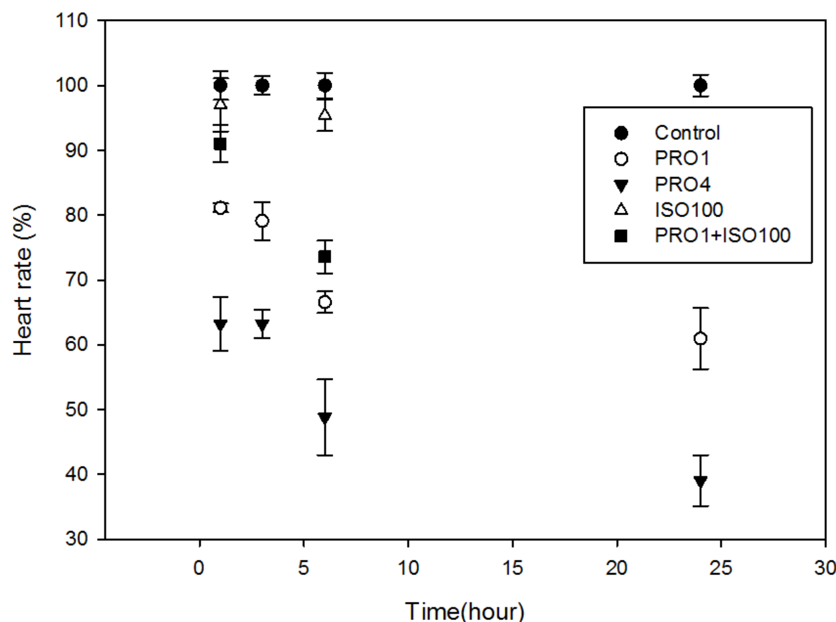
Figure 2. (a) Heart size, (b) contraction capacity, and (c) heart rate after 6-day exposure. Each bar and line represents the average and ± standard error. *P ≤ 0.05. Propranolol and Isoprenaline were 96 µg/L and 1,775 µg/L, respectively.

231 *3.2. Heart rate and gene expression profile after 24 h exposure*

232

233 Time series effects of PRO and ISO on gene expression were monitored with timely
234 synchronized heart rate measurement. The decrease in heart rate in *D. magna* was time-
235 dependent as well as dependent on types of exposures (Figure 3). PRO at 3.6 mg/L had the
236 biggest effect on heart rate among all exposure conditions and the lowest heart rate was
237 observed at 24 h. ISO did not have a significant effect, and the mixture of ISO and PRO
238 resulted in a decrease in heart rate. However, this effect was smaller than that of PRO alone.

239



240

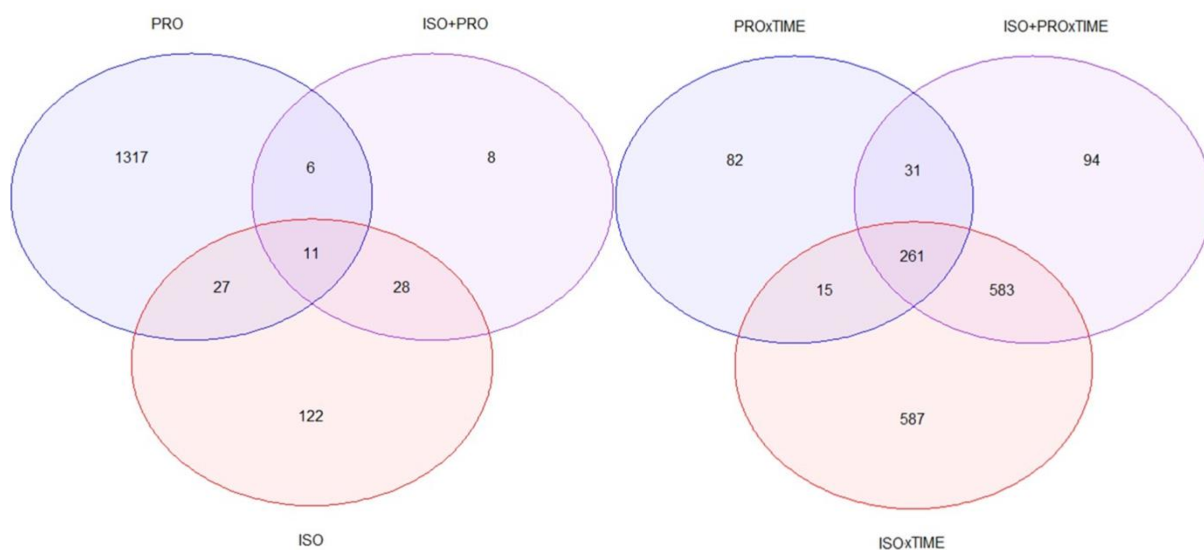
241 **Figure 3.** Heart rate measurements for the different exposures at the different time points.
242 Each scatter and line represent the average and ± standard error. Propranolol was 0.9 and 3.6
243 mg/L for PRO 1 and 4, and Isoprenaline was 84.4 mg/L for ISO 100, respectively.

244

245 At the gene expression level, we studied more than 19,000 genes and observed significantly
246 different regulation of genes across exposures and time points. When comparing
247 differentially expressed genes across the different statistical contrasts (Figure 4), we observed
248 a similarity between the different contrasts. Eleven and 261 genes were shared across the
249 different main contrasts and different interaction contrasts with time, respectively. These
250 overlapping genes between the contrasts imply that gene expressions and consequent
251 physiological changes might be partly overlapped as results of those gene expressions. In
252 figure 4, the effects of PRO on gene expression seem to be more stable across different time

253 points as we observed more than 1300 genes significantly expressed at all time points (PRO)
254 whereas we observed roughly 400 genes which were significantly expressed depending on
255 the time point (PRO X TIME). We observed the opposite for ISO and ISO + PRO. For those
256 treatments, gene expression depends on the time point as more genes were significantly
257 expressed in ISO X TIME and ISO + PRO X TIME contrast than the main effects. Based on
258 these observations, it is assumable that ISO and ISO + PRO showed different level of
259 influence dependent on time than PRO.

260



261

262 **Figure 4.** Venn diagram of differentially expressed genes shared across different contrasts
263 regardless of time (left): genes that differ significantly between control and PRO exposures
264 regardless of time (PRO), between control and ISO exposures regardless of time (ISO), genes
265 that show a significant interaction in a combined exposure of propranolol and isoprenaline
266 regardless of time (ISO + PRO). Venn diagram of differentially expressed genes shared
267 across different time contrasts (right): genes that showed a significant interaction between
268 ISO and time (ISO X TIME), genes that showed a significant interaction between PRO and
269 time (PRO X TIME), genes that showed a significant interaction between ISO and PRO
270 exposures across time (ISO + PRO X TIME).

271

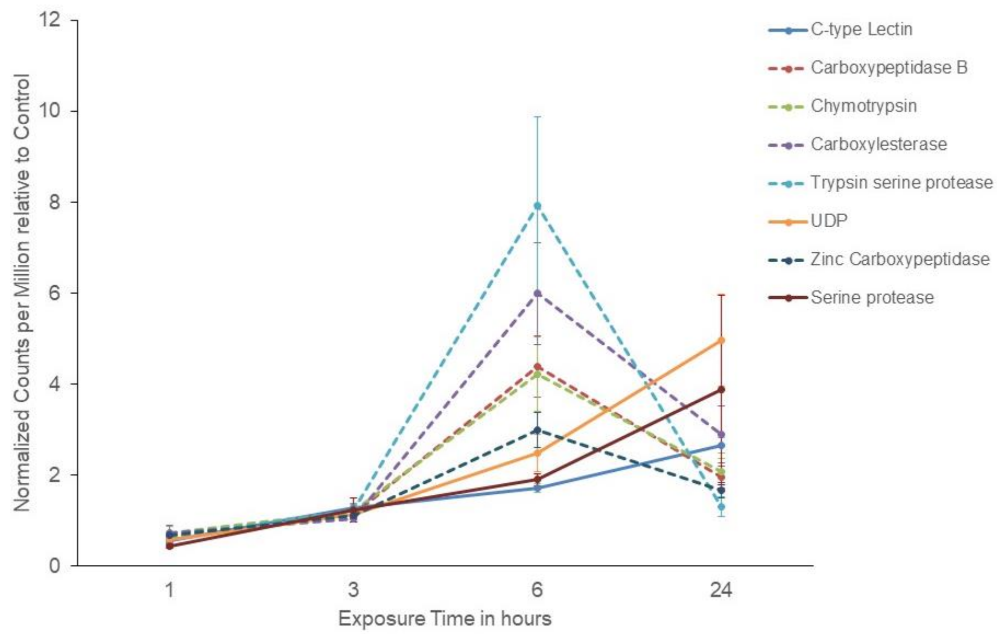
272 The known main target of PRO and ISO is the β-AR, meaning that the changes in gene
273 expression are not directly regulated by agents; however, it could provide hints on potential
274 MOAs by comparing with results of previous studies focusing on effects of β-AR activity-
275 related agents. Table 1 compares MOA-related or frequently reported gene expressions from

276 the present and previous studies. If there were too many genes annotated in a gene function to
277 be summarized, only differentially expressed genes were listed in the table. As shown in
278 Table 1, gene expression results are partially matched to the previous studies; the kinds of
279 differentially expressed genes are quite similar but the patterns of gene expressions are far
280 different. The details of all genes differentially expressed are provided in Table S2.
281
282 **Table 1.** Differential gene expressions in this study and previous studies. Genes from
283 previous studies are all regulated by β-AR activity-related agents. Up and Down arrows
284 indicate up and downregulation of gene expression. ND: Differential expression not detected.

Related function	Observation			References		
	Gene	Expression-Agent (across time)	Expression-Agent (regardless of time points)	Gene	Expression - Agent	Tissue
β-Adrenergic receptor	β-AR kinase	ND	ND	β_1-AR	↑ ↓ - ISO	Medaka heart (Kawasaki et al., 2008)
	β_2-AR	ND	ND			
Protein Kinase A	Camp-dependent protein kinase catalytic subunit	↑ - ISO ↓ - ISO+PRO	↓ - PRO			
Myosin	Myosin light chain kinase, smooth muscle	↑ - ISO, PRO ↓ - ISO+PRO	↓ - PRO	Myosin XVIIIA	↓ - PRO	Minnow brain (Lorenzi et al., 2012)
	Myosin-RhoGAP protein	↑ - ISO ↓ - ISO+PRO	↓ - PRO			
	Unconventional Myosin 16	↑ - ISO ↓ - ISO+PRO	↓ - PRO	Myosin light chain	↓ - PRO	Burned patients muscle (Herndon et al., 2003)
	Myosin 3	↓ - ISO				
Actin	α -Actinin-1	↑ - ISO ↓ - ISO+PRO	↓ - PRO	skeletal α-actin	↑ - ISO	Rat ventricular myocytes (Bishopric et al., 1992)
Apoptosis	B-cell lymphoma/leukemia 11A	↓ - ISO+PRO	ND	Caspase 8	↓ - PRO	Minnow brain (Lorenzi et al., 2012)
	Tumor necrosis factor ligand superfamily member	↑ - ISO	↓ - PRO	Caspase 3	↓ - PRO	
	Calcium/calmodulin dependent protein kinase	↑ - ISO ↓ - ISO+PRO	↓ - PRO	TGF-β3	↓ - ISO	Rat cardiac fibroblasts(Colombo et al., 2001)
Immediate early gene	c-Fos	ND	ND	c-Fos	↑ - ISO+CHT	Rat myocardial cell, Rat ventricular tissue (Brand et al., 1993; Iwaki et al., 1990)
				c-Jun	↑ - ISO+CHT	Rat

						myocardial cell (Iwaki et al., 1990)
				Jun-B	↑ - ISO	Rat ventricular tissue (Brand et al., 1993)
	Early growth response	ND	ND	Jun-D	↑ - ISO	Rodent parotid gland (Ten Hagen et al., 2002)
				Early growth response 1	↑ -ISO+CHT	Rat myocardial cell (Iwaki et al., 1990)

Apart from the expression analysis on the single genes, we observed the enrichment of several gene families with differentially expressed genes in ISO, PRO, and mixture exposures (Table S1, Figure 5, 6 and 7). Given the small number of genes for the main ISO and ISO × PRO contrasts, few significant enrichments could be detected (Table S1, Figure 5, 6 and 7), and, as such, gene family enrichment for the contrasts without time interactions will not be discussed further. Eight gene families were significantly enriched with differentially expressed genes across the different exposures in time x exposure interactions (Table 2, Figure 5, 6 and 7). For all gene families, the largest upregulation was observed after 6 or 24 h for the PRO exposure as shown in the case of PRO exposure (Figure 5). In addition, we observed dose-dependent gene expression after 6 h for all these gene families for the different PRO exposures (Figure 6). For all gene families, the gene expression patterns can be described as similar for all exposures. Only C-type lectin and carboxylesterase gene families showed different regulation patterns by the mixture of PRO and ISO at 6 h, suggesting a potential mixture interaction between the effects of ISO and PRO at the gene level.



301

302 **Figure 5.** Average gene expression patterns for different gene families in PRO 0.9 mg/L
303 relative to control exposure across four time points. (e.g., Value 1 means that normalized
304 counts per million are equal in propranolol and control exposures). Error bars represent
305 standard errors.

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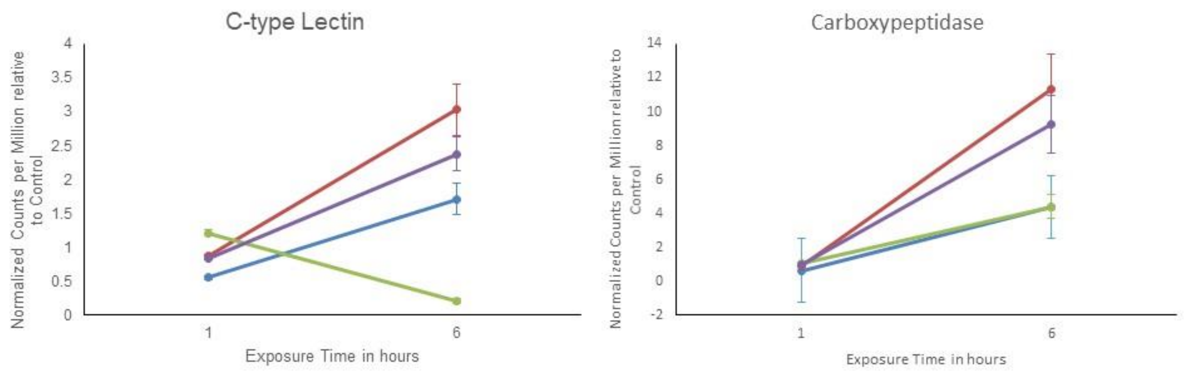

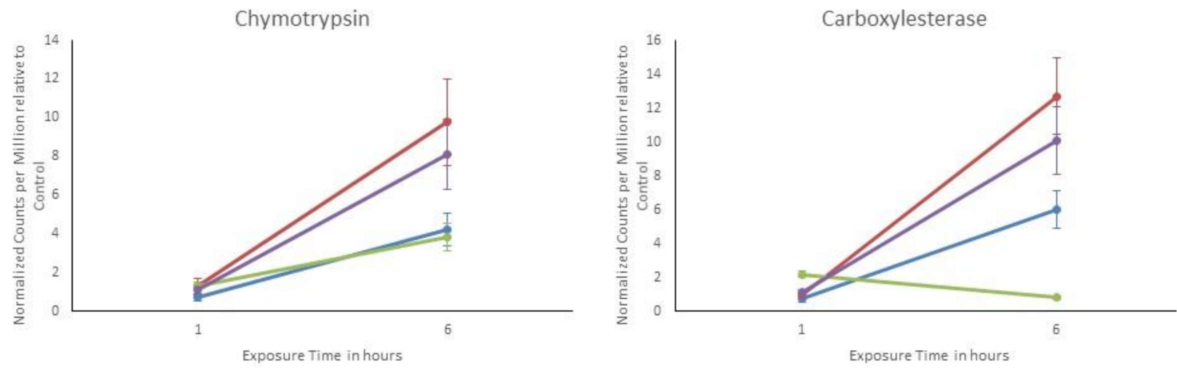

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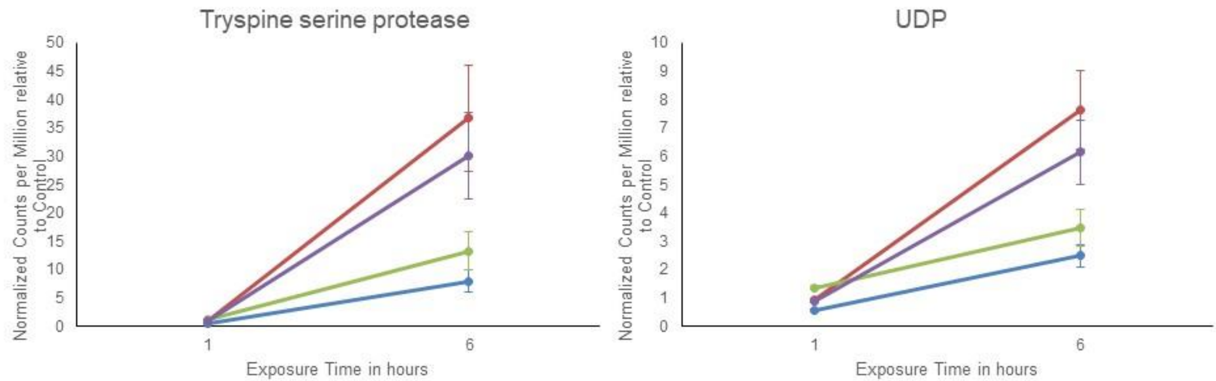
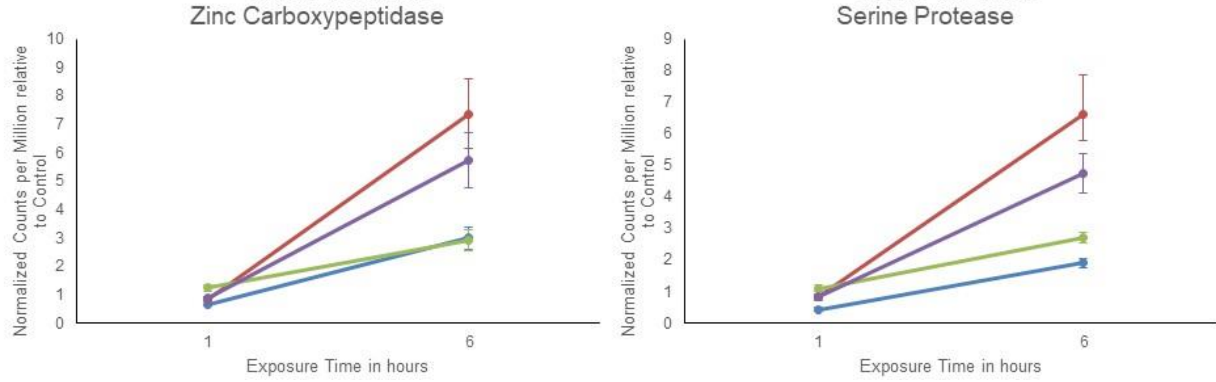




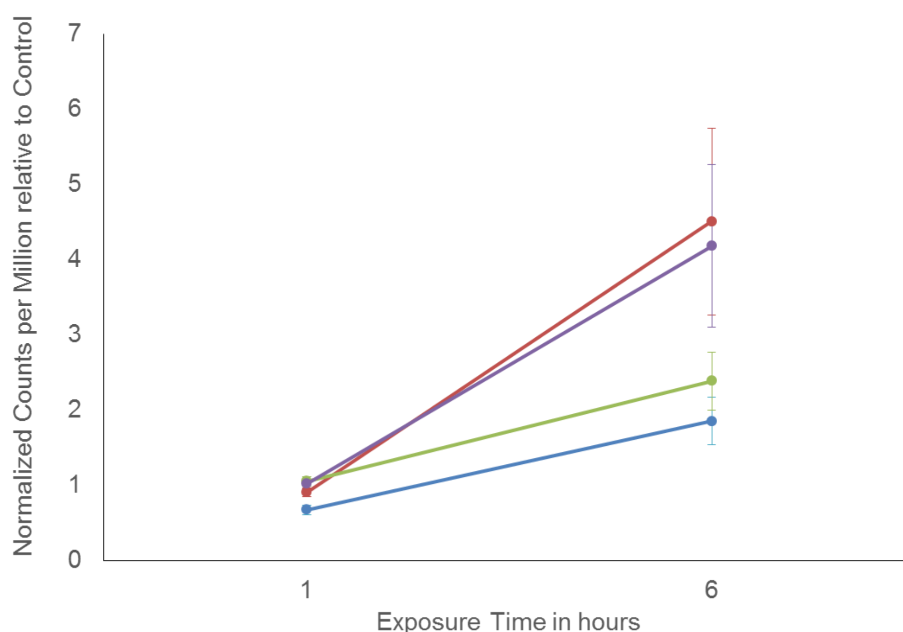
310

311 **Figure 6.** Average gene expression patterns for different gene families in PRO 1 mg/L (blue),

312 PRO 4 mg/L (purple), ISO 100 mg/L (red), PRO 1mg/L + ISO 100 mg/L (green) across two

313 time point.

314 Among the gene families significantly enriched, only 2 gene families were enriched by ISO
315 and PRO individually, but not by ISO + PRO. Surprisingly, these 2 gene families showed a
316 time-dependent pattern for ISO, while they showed a consistent pattern over time for PRO
317 (Figure 7, Table S1). Indeed, ISO exposure across time regulated genes, which have
318 endocrine disrupting (ED) potential, the juvenile hormone inducible proteins while this was
319 regulated significantly but consistently regulated over time in PRO (Figure 7, Table S1).
320 Similarly, the mRNA capping enzymes are significantly enriched in the ISO X TIME and in
321 the PRO contrast, but not in any others. These genes are involved in gene expression and
322 splicing. While it seems likely that exposure to toxicants affects gene expression and splicing,
323 it is unclear as to why these effects are observed for the single stressors but not for the
324 combined treatment.



325

326 **Figure 7.** Average gene expression patterns for the juvenile hormone inducible proteins in
327 PRO 1 mg/L (blue), PRO 4 mg/L (purple), ISO 100 mg/L (red), PRO 1mg/L + ISO 100 mg/L
328 (green) across two time points. Error bars represent standard errors.

329

330 **4. Discussion**

331

332 *4.1. Effect on cardiac structure and function after 6-day exposure*

333

334 Heart size is known to be affected by β-AR activity (Osadchii, 2007; Stanton et al., 1969).
335 Abnormal enlargement of the heart, or cardiomegaly, has been reported to be induced by β-
336 AR activation via ISO administration in rats (Osadchii, 2007; Stanton et al., 1969). Both PRO
337 and ISO have demonstrated negative and positive effects in the heart size of fish (Kawasaki
338 et al., 2008). Here, the size of the heart of *D. manga* was not affected (Figure 2a). In addition,
339 the results of contraction capacity were not comparable to previous studies (Figure 2b). In
340 general, cardiac output, which is a function of the heart rate and stroke volume, is known to
341 be increased by ISO (McQueen et al., 2005) with increases in both heart rate and stroke
342 volume (Fenyvesi and Hadházy, 1973; Kouchoukos et al., 1970). Because the stroke volume
343 is the amount of blood per heartbeat, the contraction capacity must be positively related to the
344 stroke volume. As the reduction of the contraction capacity in this study implies a potential
345 reduction in stroke volume, the effect caused by ISO contrasts with the results of previous
346 studies, in which an increase in heart rate and stroke volume were reported.

347 ISO and PRO are known to have opposite regulatory effects on the heart rate in humans,
348 and PRO is able to negate the effect of ISO (Brick et al., 1968). The decreased heart rate in
349 the PRO exposure group, and the decreased but higher level than that of PRO alone in the
350 mixture, supports a similar action of the chosen drugs in *D. magna* (Figure 2c, 3). On the
351 other hand, no change in ISO exposure suggests a weak binding affinity or a difference in
352 pharmacodynamics of ISO in the heart of *D. magna*. Berghmans et al. reported a non-
353 significant mild increase of zebrafish heart rate at 1 mM of ISO exposure; otherwise, gut
354 contraction was severely affected in the same exposure condition (Berghmans et al., 2008).
355 Their results are comparable to ours in terms of the influence of ISO on certain biological
356 functions, but not heart rate, in non-targeted organisms.

357 Overall, the observed responses in *D. magna* hearts highlight clear differences from the
358 known actions of PRO and ISO. We observed effects partially identical to the
359 pharmacological effects on heart rate, but the observations on heart size and contraction
360 capacity were totally unpredictable from the known MOA. It is assumed that the discrepancy
361 in actions of the APIs results from structural differences of target receptors or variations in
362 the distribution and function of target receptors between species. Such species deviations
363 have been reported; for example, β_2-AR activation induces a positive inotropic response in
364 myocytes of cats and dogs, but not in guinea pigs, due to variations in receptor distribution
365 and physiological function (Booze et al., 1989; Steinberg, 1999). Different amino acid
366 sequences of target receptor subtypes could also contribute to functional differences (Finch et

367 al., 2006; Michel and Insel, 2006). Because these differences occur between relatively closely
368 related mammals, it is not surprising that differences exist between mammals and *D. magna,*
369 and this supports a predicted low similarity of β_2-AR between *Daphnia* and humans based on
370 genome sequence data (Gunnarsson et al., 2008).
371
372 *4.2. Effect on whole-body gene expression during 24 h exposure*
373
374 In figure 4, more stable effects on gene expression were observed across time in PRO than
375 ISO and ISO + PRO. This may suggest that the pathways triggered by PRO require
376 continuous expression whereas the pathways triggered by ISO and ISO + PRO require
377 subsequent expression of different genes over different time points. Temporal patterns for
378 gene expression have already been observed for metallothionein genes in response to metal
379 exposure most likely due to the long half-life of metallothionein proteins (Asselman et al.
380 2013). As such, this could also suggest that genes regulated by PRO encode for proteins with
381 a shorter half-life, thus leading to more continuous RNA expression, while genes regulated
382 by ISO and ISO + PRO encode for proteins with a long half-life, thus requiring only RNA
383 expression at specific time points.
384 Table 1 shows similar genes were affected in expression but the patterns of gene expression
385 were different compared to the previous studies. This tendency is in the same context of the
386 observed impacts on the cardiac endpoints in this study. β-AR activity has been known to be
387 involved to muscle contraction, cell growth, apoptosis and a variety of other functions in
388 different organs, particularly in the heart (Communal et al., 1998; Devic et al., 2001; Simpson
389 et al., 1991). In the major signaling pathway, stimulation of β-AR results in a signaling
390 cascade sequentially consisting of G protein-mediated adenylyl cyclase activation, c-AMP
391 generation, PKA activation, and phosphorylation of diverse proteins, which leads to
392 physiological changes (Perez, 2006). β_1-AR gene, a gene of the target receptor of PRO and
393 ISO, did not influence the expression, unlike the up- and downregulation by ISO in the fish
394 model (Kawasaki et al., 2008). Cyclic adenosine monophosphate (cAMP)-dependent protein
395 kinase (PKA), a major downstream enzyme, phosphorylates various substrates in the AR
396 signaling pathway; for example, the L-type Ca_2^+ channel is phosphorylated by PKA and the
397 increased inner cell Ca2+ concentration of myocytes (Perez, 2006). Expression of the PKA
398 catalytic subunit gene was up- and downregulated by agents and its mixture in the present
399 study. Myosin-related gene expressions were reported to be downregulated by PRO in fish

400 and burned patients (Herndon et al., 2003; Lorenzi et al., 2012). In the present study, several
401 myosin-related gene expressions were significantly regulated by drug exposures; a mixture
402 and solutions of PRO and ISO up and downregulated most of the gene expressions, except
403 Myosin-3, which is downregulated by ISO only. Actin is supposed to be related to muscle
404 cell morphological and developmental regulation along with myosin. A study found that gene
405 expression of the skeletal α-actin is upregulated by ISO in rat ventricular myocytes
406 (Bishopric et al., 1992). Expression of the α-actinin-1 was found to be up and downregulated
407 by the mixture and single solutions of PRO and ISO. Apoptosis mediated by β_1-AR activation
408 has been reported (Communal et al., 1999) and in-vitro studies have revealed that myocyte
409 apoptosis results from PKA-independent activation of calcium/calmoduline-dependent
410 kinaseⅡ (CaMKⅡ) (Zhu et al., 2003). Differential expressions of the gene related to
411 apoptosis and CaMKⅡ are detected in the present study, but the impacts on gene expression
412 were not comparable to those of previous studies. Additionally, early response gene
413 expressions were compared to those of the previous studies; however, there was also lack of
414 similarity.

415 Obviously, there are limitations in the comparison of gene expression between studies. The
416 studies used different organisms, tissues, effect concentrations, and exposure times.
417 Complexity of the MOA of agents is another factor making the translation of gene expression
418 results challenging; for example, ISO and PRO also targets mitogen-activated protein kinase
419 1 (MAPK1), phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R,
420 phosphodiesterase 4 (PDE4), superoxide dismutase 1 (SOD1), and 5-HTR1, which share so
421 many molecules in their cascade signaling pathways with β-AR pathway (Masson et al., 2012;
422 Wishart et al., 2017). Although there are uncertainties caused by the limitations, it seems
423 clear that PRO and ISO affect genes involved in the AR pathway, and the patterns of gene
424 expressions are quite different from the previous studies, as shown in Table 1, which is in the
425 same context of physiological monitoring results because the results are only partially
426 comparable to those of other organisms.

427 Table 2 shows that most of the gene families that were significantly enriched are related to
428 protein metabolism. Carboxypeptidase (Carboxypeptidase B and Zinc Carboxypeptidase) is
429 responsible for the hydrolytic cleavage of a peptide bond at the C-terminal of protein or
430 polypeptide (Christianson and Lipscomb, 1989). Serine protease (serine protease, trypsin
431 serine protease, and chymotrypsin) also cleaves peptide bonds; trypsin-like and

432 chymotrypsin-like protease respectively target basic and non-polar amino acids (Ovaere et al.,
433 2009). Those protein-degrading enzymes are involved in a wide range of biological functions,
434 including digestion, immune response, reproduction, and protein post-translation
435 modification (Hedstrom, 2002). According to a recent observation, PRO exposure caused a
436 depletion of free amino acids in the *D. magna* metabolome (Jeong et al., 2018). The present
437 results are not sufficient to suggest which specific mechanisms result in the gene family
438 enrichments; however, as the peptide-cleaving enzymes are related to the protein metabolism,
439 the enrichment of the 5 gene families in this study may be associated with the downregulation
440 of the free amino acid contents. In addition to the carboxypeptidase and protease, the other 2
441 gene families are related to biotransformation. Carboxylesterase and UDP are individually
442 phase Ⅰ and Ⅱ enzymes (Parkinson and Ogilvie, 2001). They catalyze hydrolysis and
443 glucuronidation of xenobiotics. It was confirmed from our previous study that a major
444 metabolite of PRO in humans is also generated by *D. magna* (Jeong et al., 2016); thus, the
445 gene family enrichment related to the drug metabolism seems rational.
446
447 **Table 2.** Overview of the gene families that were enriched for differentially expressed genes
448 in statistic contrasts: (1) genes that differed significantly between PRO and control exposures
449 across time: PRO X TIME; (2) genes that showed a significant interaction between ISO and
450 control exposures across time: ISO X TIME; (3) genes that differed significantly between
451 ISO and PRO exposures across time: ISO + PRO X TIME. P values are Benjamini-Hochberg
452 adjusted P values and the result of Fisher exact test for enrichment analysis. Visualization of
453 the gene expression patterns can be found in Figures 5, 6 and 7.

	PRO X TIME	ISO X TIME	ISO + PRO X TIME
C-type lectins	9.52 e-04	5.4 e-15	9.53 e-16
Carboxypeptidase B	1.51 e-05	4.04 e-04	2.55 e-05
Chymotrypsin BI precursor	2.78 e-05	8.72 e-04	1.53 e-04
Carboxylesterase	3.00 e-09	6.22 e-08	6.66 e-10
Putative serine protease	7.68 e-06	1.24 e-04	7.63 e-06
Trypsin serine protease	1.15 e-04	8.72 e-04	9.30 e-05
UDP-glucorosonyltransferase 2A1	8.67 e-04	5.69 e-05	2.4 e-06
Zinc carboxypeptidase	8.57 e-03	2.25 e-06	6.66 e-10

The ED ability of APIs has been discussed in previous studies, and AR drugs were also mentioned as a potential ED chemical (Massarsky et al., 2011). Despite the structural similarity between PRO and ISO, only ISO showed time-dependent impacts on ED-related gene expressions (Figure 7). It seems needed to be further studied about the effect of ISO on *D. magna* requires further study, as ISO showed distinguishable and significant impacts on cardiac endpoints along with the ED-related gene expression.

5. Conclusion

Our results highlight that effects of PRO and ISO on a non-target species, *D. magna*, is unpredictable, as indicated in the available pharmacological database. Cardiac endpoints and gene expression in *D. magna* were affected by PRO and ISO in a manner similar to that of other organisms, but the results do not seem to be extrapolatable based on the results of other species. Furthermore, enrichment analysis indicated that AR drugs affect to expression of genes involved in protein metabolism, drug metabolism and, more importantly, endocrine system disruption which suggests needs for future studies for ED potential of ISO and PRO. Although this study still has limitations in study design to reveal MOAs precisely, at the same time, it proves the novelty of co-employment of physiological and transcriptional measurements in the investigation of impacts on APIs in non-target species study.

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7. Author Contributions

TJ and SDK designed the study. TJ executed all the experiments and collected all the samples. TJ analyzed the physiological data. TJ, JA, KDS, and FVN designed the gene expression experiment. JA analyzed the sequencing data with input from TJ, KDS and FVN. TJ wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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